Original Article

Preparation and characterization of poly (hydroxy butyrate)/chitosan blend scaffolds for tissue engineering applications

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Abstract

Background: Poly (hydroxy butyrate) (PHB) is a biodegradable and biocompatible polymer with good mechanical properties. This polymer could be a promising material for scaffolds if some features improve.

Materials and Methods: In the present work, new PHB/chitosan blend scaffolds were prepared as a three-dimensional substrate in cartilage tissue engineering. Chitosan in different weight percent was added to PHB and solved in trifluoroacetic acid. Statistical Taguchi method was employed in the design of experiments.

Results: The Fourier-transform infrared spectroscopy test revealed that the crystallization of PHB in these blends is suppressed with increasing the amount of chitosan. Scanning electron microscopy images showed a thin and rough top layer with a nodular structure, supported with a porous sub-layer in the surface of the scaffolds. *In vitro* degradation rate of the scaffolds was higher than pure PHB scaffolds. Maximum degradation rate has been seen for the scaffold with 90% wt. NaCl and 40% wt. chitosan.

Conclusions: The obtained results suggest that these newly developed PHB/chitosan blend scaffolds may serve as a three-dimensional substrate in cartilage tissue engineering.

Key Words: Blend, chitosan, poly (hydroxy butyrate), salt leaching, scaffold, tissue engineering

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INTRODUCTION

Tissue engineering is recently being developed to supply the increasing demand for human tissue and organ substitutes. The creation of such substitutes requires a three-dimensional, porous, biocompatible, and biodegradable scaffold.[1] Tissue engineering scaffolds should have suitable geometries to direct new tissue formation and mass transportation. For enhanced control over porosity and pore diameter as compared to most fabrication methods, a solvent-casting and particulate-leaching technique was developed. This technique involves casting a dissolved polymer around a suitable porogen, drying and solidifying the polymer, and leaching out the porogen to yield a polymer scaffold with an interconnected porous network.[1,2]

Another important thing in fabricating a scaffold is properly choosing the polymer constitutive. Poly (hydroxy butyrate) (PHB) is one of the natural

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polymers which have been studied extensively to fabricate scaffolds. Microbial PHB is a biodegradable polymer with a good biocompatibility and good mechanical properties. However, PHB has several intrinsic deficiencies in use as scaffolds, including brittleness, thermal instability in molten state, slow rate of degradation, and acidic degradation products.[3-7] One of the considering ways to modify imperfections of this polymer is combining it with other polymers. Based on studies, combining with natural polymers could be a promising option. [3-7]

Chitosan is a linear biopolymer and has been of enormous interest because of many advantages including biocompatibility, biodegradability, hydrophilicity, nontoxicity, and anti-microbial activity.[5,6] However, mechanically inferior feature of chitosan has limited its usage. On the basis of above-mentioned characteristics and properties, it can be seen that chitosan and PHB have mutual complementary potentials. Therefore, it is reasonable to expect that their individual deficiencies would be overcome if PHB and chitosan could be well blended together.[8] However, some difficulties have been frequently encountered on blending PHB with chitosan due to two main problems: (1) Melt processing technique cannot be applied since chitosan has high melting point and PHB will start to decompose before melting chitosan; (2) there are a very few common solvents available for chitosan and PHB.

The current investigation aims to prepare PHB/chitosan blend scaffolds for tissue engineering applications. The blend scaffolds were prepared using trifluoroacetic acid as a new co-solvent, combined with salt-leaching technique. Statistical Taguchi method was employed in the design of experiments. Four levels of chitosan and two levels of PHB and salt contents were chosen. According to Taguchi's L8 orthogonal array, eight PHB/chitosan blends were prepared using solution/salt leaching method. Immersion precipitation method was used in preparation of the blend scaffolds. Morphology, hydrophilicity, and degradation of the scaffolds were investigated.

MATERIALS AND METHODS

The poly (3-hydroxy butyrate) powder (molecular weight [Mw] =300,000; CAS number = 3-00-26063) was supplied by Sigma Aldrich, USA. Medium molecular weight chitosan with de-acetylation degree of 75–85% and trifluoroacetic acid with 99% purity were also purchased from Sigma Aldrich, USA. Salt with 99/9% purity was obtained from Merck, Germany, and sieved to separate 250–300 μm salt particles. Lien *et al.* pointed that rat joint cartilage in scaffolds with particle size of 250–500 μm presented better cell propagation and extracellular matrix creation.[9] Soda with purity of 98% was provided from Tetrachem Co., Iran, and solved in distilled water to obtain a saturated solution. Phosphate-buffered saline (PBS) was bought from Haymedia, India, and solved in distilled water to get a solution with a pH value of 7.2 ± 0.2.

Design of experiments

Design of the experiments was performed by the statistical Taguchi method with Qualitek 4 software. For the amount of salt, two levels (80% and 90%) have been considered because lower than 80% salt is not good for cell propagation and higher than 90% decreases mechanical strength of scaffolds. Chitosan content and PHB concentration varied in four and two levels, respectively. Moreover, the effects of each factor on physical and mechanical properties and blend morphology were studied. Eight compounds were prepared according to a L8 Taguchi orthogonal array which has eight combinations of levels. Table 1 shows the formulation of the compounds and variable factors.

Sample preparation

In the first step, weighted chitosan, according to design [Table 1] was loaded into a 25 ml balloon, and 10 ml of trifluoroacetic acid was added and mixed using a magnetic stirrer at 30°C and 400 rev/min for 4 h. Then, PHB was added to the chitosan solution and stirred. After 2 h, salt was added and mixed for 15 min. After that, the mixture was poured to a Petri dish for predrying. The predried polymeric film was immersed in saturated soda solution to emit the remained trifluoroacetic acid (TFA) solvent through immersion precipitation method. Then, the film was dried at 25°C for 24 h. After complete drying, samples were washed with de-ionized water to dissolve salt particles in the water completely. Exclusion of salt particles from the scaffold creates porosities with specific sizes. Samples were finally dried in a vacuum oven at 30°C and 50 mbar for 24 h.

Sample characterization

- Fourier-transform infrared spectroscopy (FT-IR) was performed to investigate blend crystallinity

Table 1: L8 orthogonal array of the Taguchi method, design of the eight compounds, and variable factors

Compound number	Sample code	Salt (%)	Chitosan (%)	PHB concentration (g/10 ml)
1	P3-C10-80	80	10	0.3
2	P4-C10-90	90	10	0.4
3	P3-C20-80	80	20	0.3
4	P4-C20-90	90	20	0.4
5	P4-C30-80	80	30	0.4
6	P3-C30-90	90	30	0.3
7	P4-C40-80	80	40	0.4
8	P3-C40-90	90	40	0.3

PHB: Polyhydroxybutyrate

and study the specific interactions between PHB and chitosan. It was carried out using Tensor 27 FT-IR (Bruker, Germany) equipped with ATR

- Porosity of the samples was calculated through immersion of samples in deionized water for 12 h and using equation (1)

$$P = 100\ (W - W_0)/V \quad (1)$$

where P, W, W_0, and V are porosity percent, sample weights after and before immersion in water, and sample volume, respectively.

- Scanning electron microscopy (SEM) micrographs were obtained on a Soren Technology AIS2100 SEM, Korea. Samples were broken in liquid nitrogen, and a thin coating of gold was applied on the surface of the samples to create a conductive surface
- Hydrophilicity property of the samples was investigated through two methods:

a. Contact angle of water droplet

A de-ionized water droplet was located on the sample surface using a 10 μL syringe and photographed after 5 s. The photograph was then analyzed with ImageJ software (developed by Wayne Rasband from National Institutes of Health) and the contact angle was reported.

b. Water adsorption measurement

Samples with dimensions of 1 cm × 1 cm were cut out from each scaffold and immersed in PBS solution. Weights of the samples were measured and recorded periodically in 4 days until it reached equilibrium. Weight percent of adsorbed water (WA) calculated from equation (2).

$$WA = 100\ (W_t - W_0)/W_0 \quad (2)$$

where W_0 and W_t are the sample weights before and after immersing in deionized water, respectively.

- Samples with dimensions of 1 cm × 1 cm were cut out from each scaffold and weighted (W_0). About 10 mL of PBS solution was poured over the samples in the Petri dish. Weights of the samples were measured and recorded periodically per 7 days (W). R, L, t, and R_t are the remained weight percent, loss weight percent, time, and mean degradation rate, respectively, which are calculated from equations (3, 4, and 5). Changes of pH in the PBS solution of samples in Petri dishes also were measured and reported.

$$R = 100W/W_0 \quad (3)$$

$$L = 100 - R \quad (4)$$

$$R_t = L/t \quad (5)$$

RESULTS

Fourier-transform infrared spectroscopy analysis

FT-IR spectroscopy result was used to study the PHB/chitosan blend crystallinity and specific interactions between the polymers. Figure 1 shows the FT-IR spectra of pure PHB and pure chitosan. The spectrum of PHB has a major characteristic absorption at about 1724/cm, which is related to stretching crystalline carbonyl (C=O) band. The absorption peak of PHB is made of at least three components at 1744/cm, 1735/cm, and 1720/cm, which belongs to asymmetric stretching vibration and the symmetric stretching vibration. Chitosan has characteristic absorptions peak at about 3400/cm, 1650/cm, and 1550/cm. The 3400/cm peak is pointed to the O–H and the symmetric stretching vibration of N–H groups, and the other two bands are related to the tension –C=O (amide I), amide II, and the N–H stretching.

Figure 2 shows the FT-IR spectra of pure PHB and PHB/Chitosan blends with 10%, 20%, 30%, and 40% wt. chitosan.

Figure 3 depicts FTIR spectra of PHB/chitosan blends with 10–40% chitosan.

Porosity measurement

Experimental and theoretical porosity percentages of the samples were calculated from equations (1) and (6), respectively. Figure 4 shows these values versus chitosan content for the scaffolds fabricated with 80% and 90% salt.

$$P = 100\ ([W \times W_s]\rho_s/W_t)/V^{[10]} \quad (6)$$

where W, W_s, W_t, ρ_s, and V are the sample weight, primary salt weight, primary polymer weight, salt density, and sample volume, respectively. The results show that with increase of chitosan, the porosity volume of scaffolds also increases (80–90%).

Scanning electron microscopy analysis

Figure 5 shows the scanning electron micrograph of top and sub-layer of the scaffolds. A thin and rough

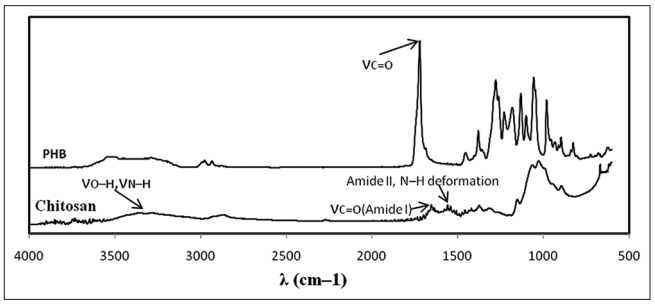

Figure 1: Fourier-transform infrared spectroscopy spectra of pure poly (hydroxy butyrate) and chitosan

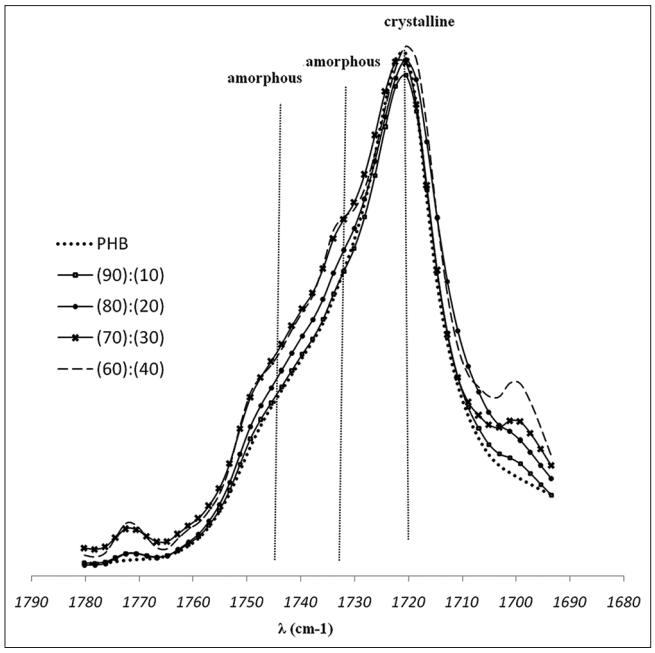

Figure 2: Fourier-transform infrared spectroscopy spectra of pure poly (hydroxy butyrate) and poly (hydroxy butyrate)/chitosan blends with 10%, 20%, 30%, and 40% wt. chitosan

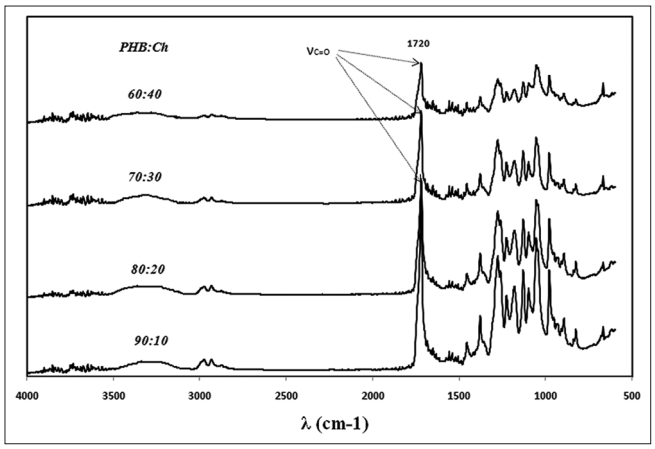

Figure 3: Fourier-transform infrared spectroscopy spectra of poly (hydroxy butyrate)/chitosan blends with 10–40% chitosan

top layer with a nodular structure, supported with a porous sub-layer in blend scaffolds. Figure 6 shows the sub-layer and cross-section micrograph of the P3-C10-80 and P4-C40-80 scaffolds. Figure 7 reveals the cross section micrograph of the P3-C30-90 and P4-C30-80 scaffolds with similar amount of chitosan and different salt content.

Hydrophilicity of the scaffolds

Figure 8 shows the contact angle of the scaffolds containing 0–40% wt. chitosan and different amounts of salt crystals. The results showed with increase in chitosan, the contact angle decreases and hydrophilicity increases.

Degradation rate measurements

In vitro degradation of the scaffolds in PBS has been studied by measuring weight of samples and PBS pH for 14 weeks. Figure 9 illustrates the percent of remaining weight of the scaffolds versus time.

PHB degradation occurs through hydrolysis and produces butyric acid which decreases the pH of the PBS solution. Figure 10 shows the pH changes in PBS solutions of samples with different amounts of chitosan.

DISCUSSION

Fourier-transform infrared spectroscopy analysis

Increasing the chitosan content increases the contribution of the amorphous fraction. This result indicates that the crystallization of PHB is suppressed by blending with chitosan. Ikejima *et al.* reported the same conclusion for PHB/chitosan blends with 10–50% wt. PHB.[4]

The IR spectrum of a semi-crystalline polymer should be made up of at least two major components from "crystalline" and "amorphous" phases.[4]

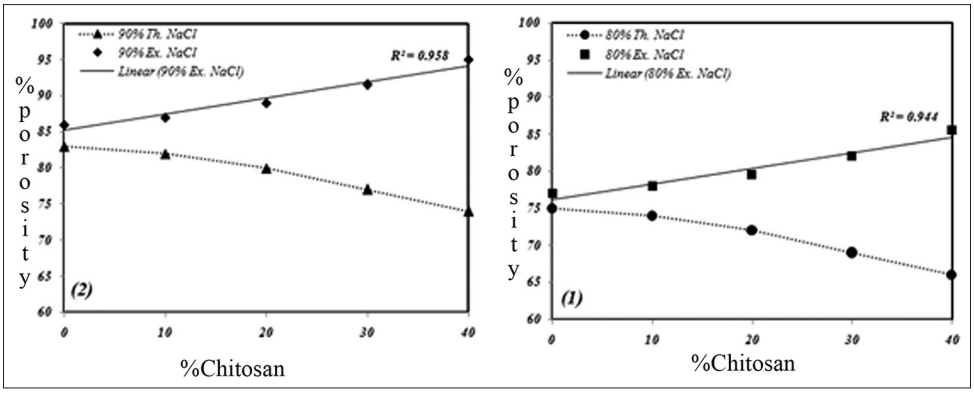

Figure 4: Experimental and theoretical porosity percent versus chitosan content for the prepared scaffolds with 80% and 90% salt

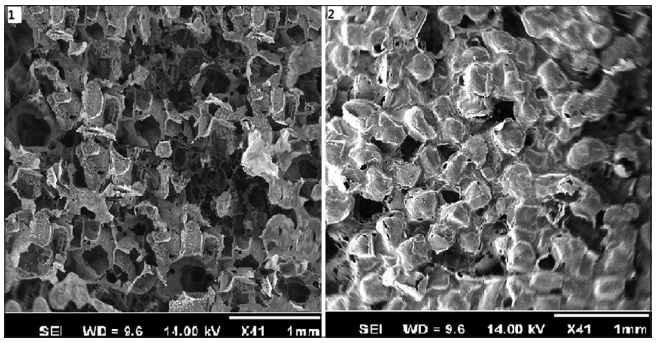

Figure 5: Scanning electron micrograph of top and sub-layer of the poly (hydroxy butyrate)/chitosan scaffolds

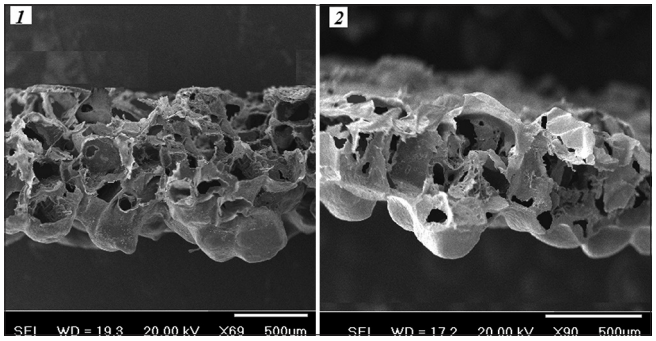

Figure 7: The cross-section micrograph of the P3-C30-90 and P4-C30-80 scaffolds with similar amount of chitosan and different salt content

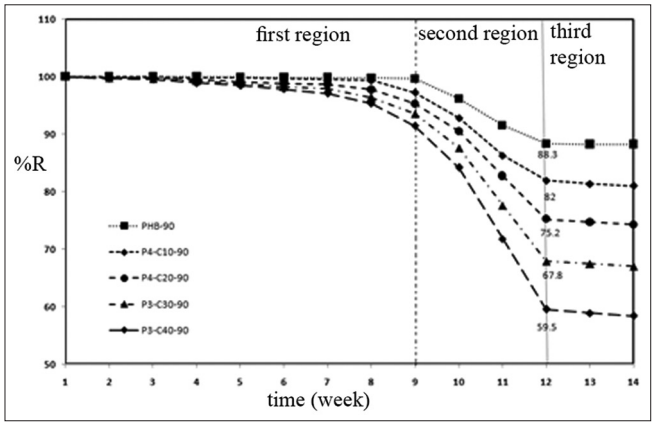

Figure 9: The percent of remaining weights of the scaffolds versus time

The lowest band is due to the crystalline phase because this band is not observed in the molten state. The other ones are related to the amorphous phase.[4]

When strong intermolecular hydrogen bands are formed between PHB and chitosan, the carbonyl band of PHB is supposed to divide into three components, i.e., crystalline, free amorphous and interacting amorphous bands. In the PHB/chitosan blends, as shown in Figure 2, no split peaks could be detected which attributed to "interacting" amorphous phase. Furthermore, C=O absorption peak of PHB in the

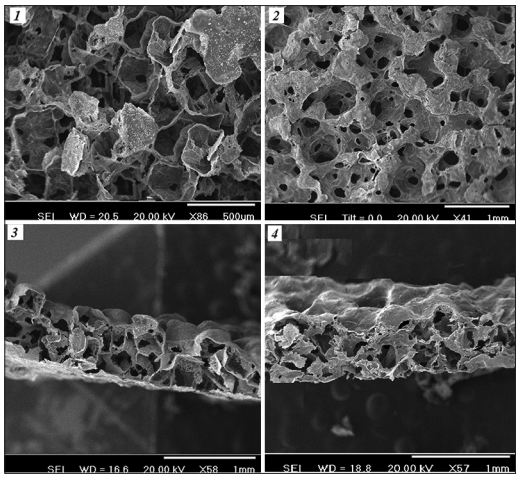

Figure 6: The sub-layer and cross-section micrograph of the P3-C10-80 (1, 2) and P4-C40-80 (3, 4) scaffolds

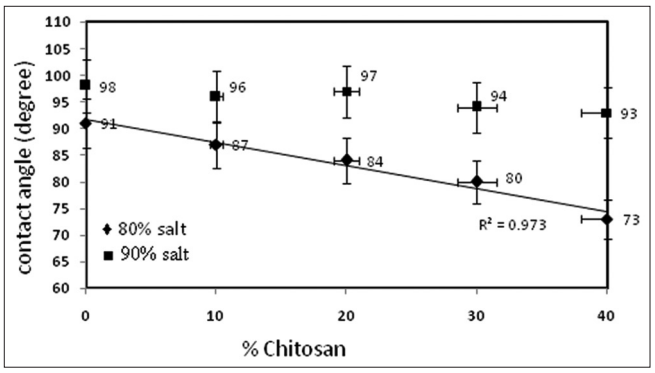

Figure 8: The contact angle of the scaffolds with 0–40% wt. chitosan and different amounts of salt

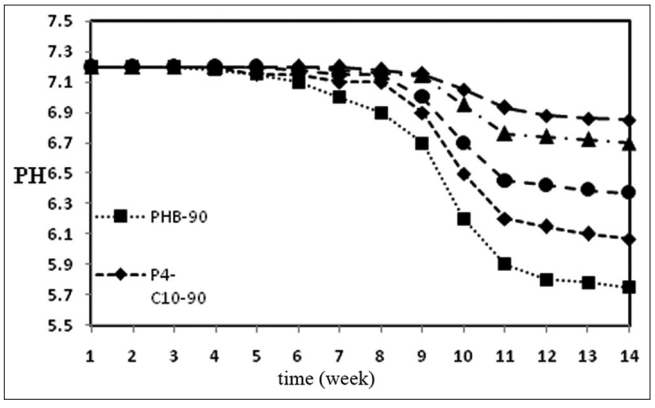

Figure 10: The pH changes in phosphate-buffered saline solutions of samples with different amounts of chitosan

PHB/chitosan blends had no significant shift to lower-wave number region.

Thus, there is no reason to expect the existence of intermolecular interaction between PHB and chitosan in the amorphous phase. Other researchers in this field reported the same conclusion.[4,11]

Porosity measurement

The theoretical and experimental porosity percent of the scaffolds increased with increasing the salt content, as shown in Figure 4. Increasing the amount of chitosan increased the experimental porosity values, whereas the theoretical values decreased. The experimental values were higher than the theoretical ones. It is because equation 6 just calculates the porosities caused by salt without considering solvent-leaching effects.

Scanning electron microscopy analysis

As SEM images suggested, there are two kinds of pore in the blend scaffolds: Cubic pores that arise from salt-leaching and small spherical pores that arise from further immersion precipitation. Increasing the amount of chitosan decreases the cubic structure of the pores and increases the interconnectivity in the scaffolds. Figure 7 illustrates the increase of porosity percent of the scaffolds with increasing the salt content. The SEM results confirm the porosity measurements data.

Hydrophilicity of the scaffolds

The contact angle of blend scaffolds were influenced by salt content and chitosan concentration; with increase in the salt content, the top layer became rougher and the contact angle increased, but increasing the chitosan concentration decreased the contact angle. In the case of 80% salt, with increasing the chitosan content from 0% to 40% wt., 20% decrease in the contact angle was observed.

Although the contact angle measurement is a good method for investigating hydrophilicity property of PHB/chitosan, it is not a perfect method for porous scaffolds as in this case, so water adsorption measurement was also carried out. However, similar results were observed for two methods.

Degradation rate measurements

Figure 9 can be divided into three regions. The first region in which the weight loss rate was low, it took 9 weeks. The second region included 9–12th week with an intensive degradation rate and in the third region, the weight loss rate was low again. Increasing the amount of chitosan increased the weight loss rate in all regions because of increasing the water diffusion possibility. The degradation rate of blend scaffolds was higher than pure PHB scaffolds.

Based on Figure 10, increasing the chitosan content (0–40%) in the blends decreased the pH reduction during the degradation. The dissolution of chitosan could neutralize the acidity of PHB degradation products.

Statistical analysis using Qualitek 4 software (from Nutek, Inc. Bloomfield Hills, Michigan, USA) showed that the salt content had most effects on porosity percentages and contact angles of the scaffolds, and the amount of chitosan and PHB concentration had second and third level of importance, respectively. The most effective factor in the water adsorption value was the amount of chitosan. The amount of chitosan and salt influenced the rate of degradation about 85% and 10%, respectively, and the PHB concentration had no significant effect. The optimum value for these properties was observed in the scaffold with 90% wt. NaCl and 40% wt. chitosan.

CONCLUSIONS

In the present work, PHB/chitosan blend scaffolds were prepared using trifluoroacetic acid as a new co-solvent with salt-leaching technique, and morphology, hydrophilicity, and degradation of the scaffolds were investigated.

FT-IR test revealed that the crystallization of PHB in these blends is suppressed when the concentration of chitosan was increased.

SEM images showed a thin and rough top layer with a nodular structure, supported with a porous sub-layer in blend scaffolds.

The contact angle illustrates that increasing the salt content, makes the top layer rougher and the contact angle increases, due to the presence of air pockets under the liquid drop,[12] but increasing the chitosan concentration decreases the contact angle, as well as the water adsorption of the scaffolds increased with increase in the chitosan concentration.

In vitro degradability investigation indicated that the degradation rate of blend scaffolds was higher than pure PHB scaffolds, and the dissolution of chitosan could neutralize the acidity of PHB degradation products. Maximum degradation rate has been seen for prepared scaffold with 90% wt. NaCl and 40% wt. chitosan.

The obtained results suggest that these newly developed PHB/chitosan blend scaffolds may serve as a three-dimensional substrate in cartilage tissue engineering.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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